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**A versatile two-step CRISPR- and RMCE-based strategy for efficient genome
engineering in *Drosophila***

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Abstract

The development of CRISPR/Cas9 technologies promises a quantum leap in genome-engineering of model organisms. However, CRISPR-mediated gene targeting reports in *Drosophila* are still restricted to a few genes, use variable experimental conditions and vary in efficiency, questioning the universal applicability of the method. Here, we developed an efficient, two-step strategy to flexibly engineer the fly genome by combining CRISPR with recombinase-mediated cassette exchange (RMCE). In the first step, two sgRNAs, whose activity had been tested in cell culture, were co-injected together with a donor plasmid into transgenic *Act5C-Cas9*, *Ligase4* mutant embryos and the homologous integration events were identified by eye fluorescence. In the second step, the eye marker was replaced with DNA sequences of choice using RMCE enabling flexible gene modification. We applied this strategy to engineer four different loci, including a gene on the fourth chromosome, at comparably high efficiencies, suggesting that any fly lab can engineer their favourite gene for a broad range of applications within about three months.

Reverse genetics is currently booming with the establishment of TALEN- and CRISPR-mediated genome engineering¹⁻³. In particular, the CRISPR/Cas9 technology appears to efficiently and specifically introduce double strand DNA breaks in the genome of the organism, which can then be utilised to either introduce point mutations by error prone nonhomologous end-joining (NHEJ) or to integrate heterologous DNA into the chromosome using the homology-directed repair (HDR) pathway². In *Drosophila*, CRISPR-induced NHEJ technology has mainly been utilised to mutate genes that result in a visible, easily scored phenotype, such as white eyes or yellow body colour, or to mutate GFP transgenes⁴⁻⁷. Mutants in genes with no visible phenotype required laborious PCR screening for their identification⁸⁻¹⁰, thus far hampering CRISPR-based gene mutagenesis to develop into a routine application. Recently, this bottleneck was addressed by applying CRISPR-induced HDR to insert an attP-site together with a visible marker into the gene of interest¹¹⁻¹³. In some cases the visible marker was flanked by FRT or loxP sites allowing its excision to only leave one attP site (and one loxP or FRT site) within the gene. This attP site enables the introduction of any given DNA sequence into the gene of interest^{11,12}. However, the efficiency of reporter integration was rather low¹¹ and only determined at a single genomic locus^{12,13}, leaving the general applicability to the *Drosophila* genome an open question.

Here, we have developed a highly flexible two-step genome engineering platform that combines CRISPR-mediated HDR with Φ C31 recombinase-mediated cassette exchange (RMCE). In the first step, CRISPR is applied to integrate a splice acceptor and an SV40 terminator together with a 3xP3-dsRed eye reporter. This enables both, the efficient identification of the targeted event and the creation of a strong loss of function allele. In the second step, two flanking attP sites are utilised to

replace the inserted DNA by any DNA of choice using RMCE, an established standard technology in *Drosophila*¹⁴. Together, this allows flexible cassette exchange to freely manipulate the gene of interest. We successfully applied this method to four different loci and efficiently generated several allele variants, including a conditional allele, from a single targeting event. Our streamlined CRISPR/Cas9- and RMCE-based strategy makes it practical to flexibly engineer any *Drosophila* gene of choice for a broad range of applications within about three months.

Results

Strategy overview

We aimed to develop a versatile and efficient strategy to modify the *Drosophila* genome, which would allow various genome modifications such as the introduction of single point mutations, protein tags, exon deletions or other desired changes in the gene of choice. Despite the suggested higher efficiency of CRISPR/Cas9-induced HDR as compared to Zn-finger- or TALEN-induced HDR, the identification of successfully targeted carrier flies is still a limiting step in the process. PCR-based screening or melting curve analysis methods require DNA extraction¹⁵ and thus are laborious and often inconvenient for efficient stock generation. Therefore, we decided to develop a 2-step strategy as illustrated in Figure 1, which enables efficient identification of the carrier flies and allows entirely flexible genome engineering. In the first step, we insert a 3xP3-dsRed marker enabling easy identification of the HDR event. A strong splice acceptor, followed by STOP codons and an SV40 polyA terminator, precedes the dsRed cassette. The inserted DNA is flanked by two attP sites in opposite orientations, a strategy that we adapted from the popular MiMIC system¹⁴. If this cassette is inserted into an intron, or replaces an endogenous exon, it results in a truncated mRNA of the targeted gene. Thus, step 1 creates a loss of function allele (Figure 1).

In the second step, RMCE is applied to replace the DNA between both attP sites by any DNA of choice, leaving a minimal scar of two attR sites, preferably in introns. RMCE has been used very efficiently in the MiMIC system, demonstrating that attR sites in introns generally do not interfere with gene function¹⁴. Hence, our strategy enables the generation of various alleles, like a defined point mutation, a tagged allele, an exon replaced by a tag or a conditional allele, from a single HDR

carrier (Figure 1). This strategy should allow flexible editing of any *Drosophila* gene within about three months (Figure 2).

CRISPR design and cloning

In step 1 we aimed to insert a STOP-3xP3-dsRed cassette flanked by two attP sites using a donor plasmid (Figure 1). Since the same strategy should be applicable to any gene, we established a single step Golden Gate protocol to assemble the STOP-dsRed donor plasmid containing about 1kb homology arms on each side, which has been shown to be of sufficient length for efficient HDR¹⁶ and can be easily amplified by PCR. Cloning of the homology arms into the donor vector is thus very straightforward and takes only a few days for the gene of choice (Figure 3a, see Methods). This donor vector is the template for the HDR in step 1.

In step 2 RMCE exchanges the STOP-dsRed by any sequence located between 2 attB-sites in a provide plasmid (Figure 1). RMCE works very reliably and a large collection of plasmids to tag genes or insert reporters for various applications is available¹⁴. These plasmids are fully compatible with our step 2 design. We have generated additional “generic” attB plasmids that can be used to tag any gene with a 2xTY1-V5 tag or to engineer a conditional allele using an FRT flanked 2xTY1 cassette followed by a V5 tag (Figure 3b). Flp mediated deletion of the 2xTY1 cassette will lead to a frame shift and can thus be used to create loss of function clones at very high efficiency, as the flip-out will occur in cis¹⁷. We have generated both constructs in all three reading frames.

CRISPR activity assay in cell culture

Many search algorithms exist to predict sgRNA target sequences for a given gene region¹⁸. However, to date there is no simple way of confirming if any of the predicted sgRNAs work efficiently. We developed such a selection assay to be able to only inject effective sgRNAs into fly embryos. We designed 12 different sgRNAs targeting different regions in the *salm* gene and synthesized the sgRNAs by a standard PCR and in vitro transcription reaction (Figure 3c). These sgRNAs were then individually transfected into Cas9 expressing S2 cells¹⁹, and their cleavage efficiency was determined with a simple T7-Endonuclease I assay (see Methods). On average, about half of the tested sgRNAs work efficiently in this assay (Figure 3d and data not shown), strongly suggesting that such a pre-selection test is useful to improve the *in vivo* success rates.

Step 1 - HDR in *Lig4* mutant embryos

To test the efficiency of inserting our STOP-dsRed cassette, we designed three donor constructs targeting different regions in the *salm* gene (Figure 4a): the first, deleting parts of exon 1; the second, inserting the cassette into intron 1; and the third, deleting exon 3. For each construct about 1 kb homology arms were cloned in the STOP-dsRed donor vector. We injected the STOP-dsRed donor as circular plasmid together with 2 plasmids each containing a U6 promoter driven sgRNA verified in S2 cells and a hsp70-Cas9 source (see Methods). We injected into *Ligase4* mutant embryos, which were reported to exhibit a higher rate of HDR than wild-type embryos^{15,16}. We injected between 700 and 1500 embryos for each of the three constructs and were able to recover 11 red eyed F1 carriers from 2 independent founders for the first intron construct and 72 red eyed F1s from 4 independent founders for the third exon deletion

construct (Table 1). This demonstrated that our strategy works in principle, but as we failed to recover the first exon deletion allele, we wanted to further improve the efficiency by using a different Cas9 source.

Step 1 - transgenic Cas9 improves HDR efficiency

A number of transgenic Cas9 flies have been recently generated, some of which have been used successfully^{6,12,13}. To test whether a transgenic Cas9 source is indeed more efficient for HDR than a source from an injected plasmid, we targeted the same loci as above, but now using *Act5C-Cas9, Lig4* flies. For this, we recombined an *Act5C-Cas9* transgene expressing Cas9 ubiquitously, including maternally in the germ-line, with the *Lig4[169]* null allele. Additionally, we removed the *white* and *3xP3dsRed* markers from the *Act5C-Cas9* transgene to obtain a *Act5C-Cas9, Lig4[169]* chromosome that is useful for the injection of our donor plasmids (see Methods). We injected 700 *Act5C-Cas9, Lig4[169]* embryos with two *in vitro* transcribed sgRNAs targeting either the 1st exon, the 1st intron or the 3rd exon of *salm* (using the same sgRNA target sequences as used above). We obtained 1, 9 and 4 independent founders producing 13, 54 and 59 F1 carriers, respectively, demonstrating that all 3 loci were targeted successfully with frequencies between 3 and 14 % per fertile G₀ (Table 1). To verify that the targeted insertion occurred correctly, we tested a total of 9 independent carriers from the 3 loci by PCR and sequencing and were able to confirm all of them.

The first step of our gene targeting strategy inserts a strong splice acceptor followed by a STOP cassette into the gene and thus should terminate transcription at this position. By design, the *salm[1st exon-dsRed]* allele additionally has a deleted ATG. As expected, the *salm[1st exon-dsRed]* allele is homozygous lethal, as well as

lethal in trans to *salm*[1], demonstrating that we indeed created a strong *salm* loss of function allele (Figure 5a). The *salm*[1st intron-dsRed] allele harbours an insertion in the first intron (Figure 4a). This allele is also homozygous lethal, and lethal in trans to *salm*[1] suggesting that the splice acceptor and STOP cassette are used efficiently to create a strong loss of function allele (Figure 5a). The *salm*[3rd exon-dsRed] allele only deletes the last 36 amino acids of the long SalmPA isoform, including 10 amino acids of the last zinc finger (Figure 4a). This allele is homozygous viable, but flightless (Figure 5a), implying a role for the last zinc finger in flight muscle function. Taken together, these data suggest that our CRISPR-mediated step 1 strategy works efficiently to isolate targeted carrier flies at a practical frequency for routine use. Conveniently, these step 1 alleles are loss function alleles if the insertion is located within the gene.

Step 2 - Flexible gene editing by RMCE

A major benefit of our editing strategy is the flexible step 2 that enables the near-seamless insertion of any DNA sequence with only two remaining attR sites (Figure 1). To test the feasibility of step 2, we chose the *salm*[1st intron-dsRed] allele. We exchanged the STOP-dsRed cassette with a short 2xTY1-V5 exon, a FRT-2xTY1-FRT-V5 conditional exon and a large GFP-3xFLAG exon from¹⁴ (Figure 4b). As expected, in all three cases the cassette exchange worked routinely (see Methods). Importantly, the *salm*[1st intron-dsRed] lethality was reverted by RMCE in all three cases (Figure 5b). This demonstrates that our editing protocol generally does not result in any unwanted harmful mutations on the edited chromosome.

Salm protein is expressed in indirect flight muscles (IFMs) and is essential for fibrillar IFM fate specification²⁰. Thus, we should detect the tagged Salm protein

versions in the IFM nuclei of adult flies. Indeed, tagged protein from all three alleles, *salm*[*Ist* intron-TY1-V5], *salm*[*Ist* intron-FRT-TY1-FRT-V5] and *salm*[*Ist* intron-GFP-FLAG] is expressed in IFMs. Salm-TY1-V5 and Salm-GFP-FLAG are readily detected in the IFM nuclei, Salm-FRT-TY1-FRT-V5 shows an additional dotted pattern in the cytosol (Figure 4c - g). The fibrillar IFMs organisation in all three homozygous *salm* alleles is normal showing that the tagged Salm proteins are indeed functional. Each IFM fiber contain several hundred nuclei. The conditional *salm*[*Ist* intron-FRT-TY1-FRT-V5] should now enable a clonal loss of function analysis of *salm* in muscle only, as flip out in cis is highly efficient¹⁷. Thus, this strategy should generally be versatile for the genetic analysis of muscle in the future.

Gene editing on the fourth chromosome

In order to demonstrate the general applicability of our gene editing strategy, we decided to apply it to an additional locus. We chose the *bent* gene, located on chromosome four, which is highly heterochromatic and thus difficult to manipulate by standard genetic tools. To our knowledge, there is only a single case reported in the literature that targeted a gene located on the fourth chromosome by classical ends-out mediated homologous recombination using long homology arms²¹. *bent* encodes for a very large gene composed of at least 46 exons that are spread across more than 51 kb of genomic DNA (Figure 5). *bent* encodes for Projectin, a titin-like protein, which is specifically expressed in muscles and essential for correct sarcomeric organisation²²⁻²⁴. It is supposedly silent in germ cells in which the targeting event must happen. We chose to delete exon 11, an exon at the beginning of the PEVK domain of Projectin²⁵, using two sgRNAs flanking the exon. Both sgRNAs tested positively in the S2 cell assay (Figure 5 and data not shown). We again used approximately 1kb homology

arms and injected the donor vector into 700 *Act5C-Cas9, Lig4[169]* embryos. We isolated 5 carriers from 1 founder out of a total of 56 fertile G₀ flies, an HDR efficiency of 1.8 %. We confirmed the *bt[11th intron-dsRed]* allele by sequencing the locus. As expected, *bt[11th intron-dsRed]* is homozygous lethal and also lethal in trans to *bt[I-b]*, a strong bent allele²³, again suggesting that the inserted splice acceptor is used effectively and transcription is prematurely terminated. Together, these results demonstrate that our CRISPR-mediated targeting strategy also works efficiently on the fourth chromosome, suggesting it can be generally applied to any locus of choice in the fly genome.

Discussion

CRISPR/Cas9 has been used successfully in many model organisms to generate mutants or to introduce targeted changes by HDR¹. In *Drosophila*, there has been no general agreement regarding which strategy works most effectively to engineer the genome. To simply mutate a gene by CRISPR/Cas9-induced NHEJ, Cas9 was either injected as mRNA^{7,9}, provided from an injected plasmid^{4,11} or provided from a transgenic source^{5,6,8}. Similarly, the sgRNA was either injected as *in vitro* transcribed sgRNA, or provided by an injected plasmid or a transgenic source. A standard protocol has not yet emerged although a number of genes have been mutated.

NHEJ can only induce small insertions or deletions. In contrast, HDR allows the defined engineering of a given gene and thus is suitable for a much wider range of applications. CRISPR/Cas9-mediated HDR has been used in *Drosophila* to insert short attP or tag sequences from single strand oligonucleotides as donors⁴ or larger cassettes including a dsRed marker cassette from a plasmid donor¹⁰⁻¹³ again using various ways of injected or transgenic sources of sgRNAs or Cas9. The injected genotype was variable, sometimes *Lig4* mutants were used^{10,12}, sometimes not^{4,11-13}. Often the detection of the targeted event required laborious fly screening by PCR^{10,26}.

Here we aimed to develop a universal and efficient CRISPR-based strategy that enables flexible genome engineering, including the insertion of large tags into the coding region of a gene or the generation of conditional alleles. This strategy should be generally applicable to most *Drosophila* genes. Our results confirmed that about 1 kb homology arms are of sufficient length to insert a large marker cassette, as has been suggested before for other loci^{10,12,13}. Thus, we could develop an efficient donor plasmid assembly protocol, which facilitates cloning of the donor vector for any gene within a few days. Additionally, our data support the value of a quick pre-testing

strategy of predicted sgRNAs in S2 cells to eliminate inefficient sgRNAs, which would likely reduce targeting efficiency *in vivo*. Conveniently, the same *in vitro* transcribed RNAs can be used for both, S2 cell transfections and embryo injections. Our results suggest that indeed a transgenic Cas9 source mediates HDR effectively in *Ligase4* mutant germ line cells. Although *Act5C-Cas9* expression is not restricted to the germ line, injections of the donor vector together with 2 verified sgRNAs led to a targeting efficiency of 2 - 14 % for the incorporation of the large STOP-dsRed cassette, even for the *bent* locus on the heterochromatic 4th chromosome. This suggests that about 50 - 100 fertile G₀ flies should be sufficient in most cases to identify positive carriers. We thus far deleted up to about 1kb of genomic sequence by HDR. Larger deletions would likely occur at reduced efficiencies, however the dsRed marker should still make it practical to find them. The straight forward identification of carriers together with our simple cloning scheme, this should easily facilitate the insertion of the STOP-dsRed cassette into the gene of choice.

Our 2-step strategy combines the advantages of both, CRISPR and RMCE, and thus allows very flexible modifications of a particular gene region with minimal effort. Multiple fluorescent and affinity tags can be easily inserted or a deleted exon can effectively be replaced by various engineered exon versions. In principle, larger gene parts consisting of multiple exons can also be deleted and replaced by modified versions. This method is particularly valuable for genes that harbour complex transcriptional control and function in many tissues such as *salm* or for genes that are exceptionally large and exhibit complex alternative splicing patterns such as *bent*. The 2-step strategy allows structure-function analysis at the endogenous locus without interfering with the regulatory regions included in introns, which cannot be achieved by simply inserting a cDNA at the transcriptional start site. The functionality of our

method was verified by the reversion of the lethality for the step 2 alleles in the first intron of *salm*. This furthermore demonstrates that both steps do not generate additional unintended changes on the chromosome. Therefore, we hope that our strategy will promote the wide application of CRISPR-mediated HDR in *Drosophila*, making it a routine tool used in every fly lab like EMS mutagenesis or P-element mediated transformation were in the last century.

Methods

Fly strains and genetics

All fly work, unless otherwise stated, was performed at 25 °C under standard conditions. The *Lig4*[169] null allele²⁷ was obtained from the Bloomington *Drosophila* Stock Center, *y*[1], *M*(*Act5c-Cas9*, [*w*+]) in *M*(*3xP3-RFP.attP*)*ZH-2A*, *w*[1118] was a gift from Phillip Port and Simon Bullock. Both markers (*w*+ and *3xP3-RFP*) were removed by crossing to heat-shock-Cre. The *y*[1], *M*(*Act5C-Cas9*)*ZH-2A*, *w*[1118] flies were recombined with *Lig4*[169] to obtain *y*[1], *M*(*Act5C-Cas9*)*ZH-2A*, *w*[1118], *Lig4*[169].

Cell culture

Drosophila Schneider 2 (S2) cells stably expressing myc-Cas9 from a ubiquitin promoter were a gift from Klaus Förstemann¹⁹. S2 cells were cultured in Schneider's *Drosophila* medium supplemented with 10 % fetal calf serum (Life Technologies) and penicillin/streptomycin (GE Healthcare). sgRNA activities were tested by transfecting 1 µg sgRNA per 24 well into the myc-Cas9 cells using Eugene HD (Promega), followed by DNA extraction and a T7-Endonuclease I assay (see supplied protocol for details).

Plasmids

CC6-U6-gRNA_hsp70-Cas9 plasmid was a gift from Peter Ducheck. pJET1.2-STOP-dsRed: attP1 and splicing acceptor (SA) were amplified with primers XZ82 and XZ83, SV40 terminator with XZ84 and XZ85, and attP2 with XZ88 and XZ89 from DNA extracted from a MiMIC fly line¹⁴; 3xP3-dsRed was amplified from a fosmid fly line²⁸ with primers XZ86 and XZ87. These fragments were cloned into pLR-HD

plasmid²⁹ by Golden-gate cloning. This assembled attP1-SA-STOP-SV40-3xP3-dsRed-attP2 cassette was amplified with primers XZ195 and XZ196 and blunt cloned into pJET1.2 to generate pJET1.2-STOP-dsRed. As this STOP-dsRed cassette is flanked by two BsmBI sites, it can be easily assembled with both homology arms (Figure 3a): each homology arm of about 1kb was amplified from genomic DNA of the target genotype with Phusion polymerase (NEB) and blunt-end cloned into pJET1.2 (CloneJET PCR Cloning Kit, Thermo Scientific). Primers used to amplify the homology arms have a 5' BsmBI site enabling Golden Gate assembly with the STOP-dsRed cassette. All primers used are listed in Supplementary Table 1. pBS-donor-backbones: pBS-GGAC-TTCT, pBS-GGAC-ATGC and pBS-CGGA-GTGC were constructed by linearizing pBluescript with KpnI and SacII, followed by amplification with primer pairs XZ150 and XZ151, XZ156 and XZ151, XZ161 and XZ162, respectively, and re-ligation. The generated pBS-donor-backbones harbour two BsmBI sites for donor plasmid assembly. pJET1.2-STOP-dsRed, pJET1.2-HA-left, pJET1.2-HA-right, and an appropriate pBS-backbone were assembled to the pBS-donor vector by Golden-gate cloning. attB plasmids: FRT-2xTY1-FRT-V5 and 2xTY1-V5 fragments were synthesized as gBlocks (IDT) and cloned into the attB plasmid for all three reading frames. CC6-U6-gRNA_hsp70-Cas9-sgRNA1,3,4,7,9: the CC6-U6-gRNA_hsp70-Cas9 vector was cut with BbsI (NEB) and the annealed sgRNA targeting oligos were cloned into it. The vas- Φ C31(3xP3-EGFP.attB) plasmid was obtained from Johannes Bischof³⁰. The attB site was removed by digestion with SpeI, followed by re-ligation.

All plasmids for embryo injections were purified with PureLink® HiPure Plasmid Midiprep Kit (Life technologies). Oligos are listed in Supplementary Table 1.

sgRNA synthesis

The sgRNA dsDNA template was produced using overlap PCR with a small amount of a common sgRNA scaffold primer, a shorter sgRNA amplification primer and a sgRNA gene specific primer that includes the T7 promoter (Figure 3c)¹⁹. All sgRNA primer sequences are listed in Supplementary Table 1. The PCR product was cleaned by Qiagen MinElute kit (Qiagen). The sgRNAs were transcribed with T7-MEGAShortscript™ Kit (Life Technologies) and purified with MEGAclean™ Transcription Clean-Up Kit (Life Technologies).

Embryo injection

Pre-blastoderm embryos of the appropriate genotype were de-chorionated and injected with a FemtoJet apparatus (Eppendorf) using self-pulled glass needles (Havard Apparatus) under standard conditions at room temperature. Injected embryos were kept for 2 days at 18 °C and the hatched larvae were collected and grown at 25 °C. For step 1 injections, pBS-donor plasmid, 2 sgRNAs and optionally the CC6-U6-gRNA_hsp70-Cas9 plasmid were mixed and diluted in water. *Lig4[169]* embryos were injected with CC6-U6-gRNA_hsp70-Cas9 plasmid (100 ng / µl) and pBS-donor plasmid (500 ng / µl). *y[1]*, *M(Act5C-Cas9)ZH-2A*, *w[1118]*, *Lig4[169]* embryos were injected with both sgRNAs (60 - 70 ng / µl, each) and pBS-donor plasmid (500 ng / µl). For step 2 injections the attB plasmid (150 ng/µl) was mixed with vasa-ΦC31 plasmid (200 ng/µl).

Immunolabelling of IFMs. Hemi-thoraces of adult *Drosophila* were prepared and stained as described³¹. rabbit anti-Salm was used at 1:50³², mouse anti-Flag (Sigma), mouse anti-V5 (Abcam) and rhodamine phalloidin (Invitrogen) were all used at 1:500.

Nuclei were visualized by embedding in Vectashield plus DAPI (Vector Laboratories) and images were acquired on a Zeiss LSM780 confocal and processed with FIJI and Photoshop.

Detailed *Drosophila* genome engineering protocol by CRISPR-RMCE

(Will be deposited at <http://www.nature.com/protocolexchange/>)

1. CRISPR-sgRNA design and donor plasmid cloning ~ 10 days

- 1.1 Verify sequence of the targeting region in the fly strain used and in the S2 cells by sequencing to identify potential polymorphisms compared to the published sequence.
- 1.2 For designing the sgRNA targeting sites, choose one of the web tools¹⁸. We used an interface designed by the Zhang lab: <http://crispr.mit.edu>
- 1.3 sgRNA production
 - 1.3.1 Generate the dsDNA template for sgRNA in vitro transcription as described in¹⁹.
 - 1.3.2 Transcribe sgRNA by T7-MEGAShortscript™ Kit (AM1354, Life Technologies). Use 150 - 250 ng template for a 20 µl reaction and at 37 °C overnight.
 - 1.3.3 Purify sgRNA by MEGAclean™ Transcription Clean-Up Kit (Life Technologies). Follow the manufacturer's protocol, and in step 3, add an equal volume of 100 % ethanol to the sample.
 - 1.3.4 Check the sgRNA integrity on a gel and measure the concentration using a photometer (Nanodrop, Thermo Scientific). The expected yield is 100 µg, which is enough for the S2 cell assay and the fly injections.

- 1.4 sgRNA activity assay in S2-cells
 - 1.4.1 Grow S2 cells in Schneider medium with 10 % FCS (Life Technologies) to 5-10 x 10⁶ cells / ml at 25 °C.
 - 1.4.2 Dilute cells to 0.7 x 10⁶ / ml and plate 1ml cells in S2 medium with 10 % FCS per well in a 24 well plate for each transfection.
 - 1.4.3 Prepare the transfection mix by diluting 1 µg sgRNA in 50 µl serum free medium and 4 µl Fugene HD mix plus 46 µl serum free medium, mix both and incubate for 45 min at room temperature.
 - 1.4.4 Add the Fugene/RNA mix to each well and mix gently by pipetting.
 - 1.4.5 After 48 - 60 hours at 25 °C harvest the cells and extract the genomic DNA by QIAamp DNA mini kit (Qiagen).
 - 1.4.6 For the T7 Endonuclease I assay amplify an about 500 bp fragment, which harbours the sgRNA targeting site with Phusion polymerase (NEB), denature and anneal the PCR product as described in³³.
 - 1.4.7 Mix on ice 10 µl annealed PCR product with 10 µl T7 Endonuclease I master mix (2 µl T7 endonuclease I buffer, 0.5µl T7 endonuclease I (5 units, NEB) and 7.5 µl water).
 - 1.4.8 Digest at 37 °C for 15 - 20 min using a PCR machine and load on a 1.5 % agarose gel immediately.
 - 1.4.9 Estimate the efficiency of different sgRNAs by comparing the band intensities of the digested and non-digested bands.
- 1.5 Generation of donor plasmid (can be done in parallel to steps 1.3 and 1.4 to save time)

- 1.5.1 Amplify left and right homology arms (about 1 kb, start as close to the sgRNA cutting site as possible) with Phusion polymerase (NEB) and clone them into pJET-1.2 according to the CloneJET PCR Cloning Kit (Thermo Scientific).
- 1.5.2 Assemble the Golden-Gate Cloning reaction (50 ng pBS-backbone, 80 ng pJET1.2-HA-left, 80 ng pJET1.2-HA-right, 80 ng pJET1.2-STOP-dsRed, 1.5 μ l 10x T4 ligation buffer, 1 μ l BsmBI (NEB, R0580), 1 μ l T4 ligase (NEB, M0202) add water to 15 μ l).
- 1.5.3 Ligate in PCR machine using the following cycles: 15 cycles of (37 °C, 5 min and 16 °C, 10 min) / 37 °C, 15 min / 50 °C, 5 min / 80 °C, 5 min / 4°C
- 1.5.4 Assemble the Plasmid-safe nuclease reaction (15 μ l ligation reaction 3 μ l 10x Plasmid-safe buffer 1.2 μ l 25 mM ATP 1 μ l Plasmid-safe nuclease (Epicentre) 9.8 μ l water).
- 1.5.4 Incubate at 37 °C for 60 min in PCR machine and transform 5 - 10 μ l in bacteria. Most growing colonies will be correct.

2. Fly step 1 - CRISPR-mediated HDR ~ 6 weeks

- 2.1 Inject 600 - 800 *Act5C-Cas9, lig4[169]* embryos with pBS-donor (500 ng / μ l) and two sgRNAs (each 60 - 70 ng / μ l, targeting close to the chosen homology arms). Collect at least 50 fertile mosaic G₀ flies.
- 2.2 Cross G₀ flies individually (at least 50 vials) either to *yw* flies or appropriate balancer flies and screen all the F₁ progeny for fluorescent red eyes using a fluorescent binocular (Leica MZ16-FA). Keep track of how of many independent G₀ founders lead to how many F₁ carrier flies.

- 2.3 Generate stocks from an individual F1 carrier by crossing to balancer flies resulting in an isogenised stock for the engineered chromosome. Verify the targeting event by PCR and sequencing.

3. Fly step 2 - Φ C31-mediated RMCE ~ 6 weeks

- 3.1 Inject a “generic” plasmid generated by¹⁴ or this study or your own custom-made gene-specific attB plasmid (150 ng / μ l) mixed with vasa- Φ C31 plasmid (200 ng / μ l) into about 200 embryos from an amplified stock generated at 2.3.
- 3.2 Cross G₀ flies individually to an appropriate balancer and screen all F₁ progeny for non-fluorescent eyes using a fluorescent binocular (Leica MZ16-FA).
- 3.3 Generate stocks from an individual F1 carrier by crossing to balancer flies resulting in an isogenised stock for the engineered chromosome. Verify the correct orientation of the RMCE by PCR (will be ~50%).

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Figure legends

Figure 1 | A 2-step method to flexibly engineer the fly genome.

Overview of the 2-step procedure. In step 1, a donor vector consisting of two attP sites (P), a splice acceptor (SA), STOP codons (yellow box, black asterisk) followed by an SV40 polyadenylation signal (pA) and a 3xP3dsRed marker are inserted upon Cas9 cleavage with two sgRNAs. The orange coding exon is excised. In step 2, Φ C31-mediated RMCE inserts any DNA sequence between the 2 attB sites (B). Examples for various engineered exons are given, resulting in attR sites (R) in introns.

Figure 2 | 2-step genome engineering timeline.

Schematic overview of the major steps of the genome engineering procedure. Details are provided in the Methods section.

Figure 3 | Cloning scheme and sgRNA activity tests.

(a) Single step golden gate assembly scheme of the STOP-dsRed donor vector cloned into a modified pBluescript backbone. (b) Scheme of the “generic” attB plasmids used in our study. A simple cloning step is sufficient to generate any gene-specific attB plasmids that can be used to replace an excised exon. (c) sgRNA synthesis scheme. (d) sgRNA activity assay of 12 different sgRNAs in S2 cells. PCR result with (bottom) or without (middle) T7-Endonuclease I treatment are shown. Digested products are marked by arrow heads and effective sgRNAs are marked by a red asterisk.

Figure 4 | Engineering of the *salm* gene.

(a) Step 1 engineering of the *salm* gene. The genomic *salm* organisation is depicted with coding exons in orange. The sgRNA targeting sites are indicated by red arrows and the resulting *salm*[1st exon-dsRed], [1st intron-dsRed] and [3rd exon-dsRed] alleles are shown. (b) Step 2 engineering of the *salm* gene. RMCE products of the *salm*[1st intron-dsRed] with three different exon cassettes are shown.

Figure 5 | Phenotypic analysis of the engineered *salm* alleles.

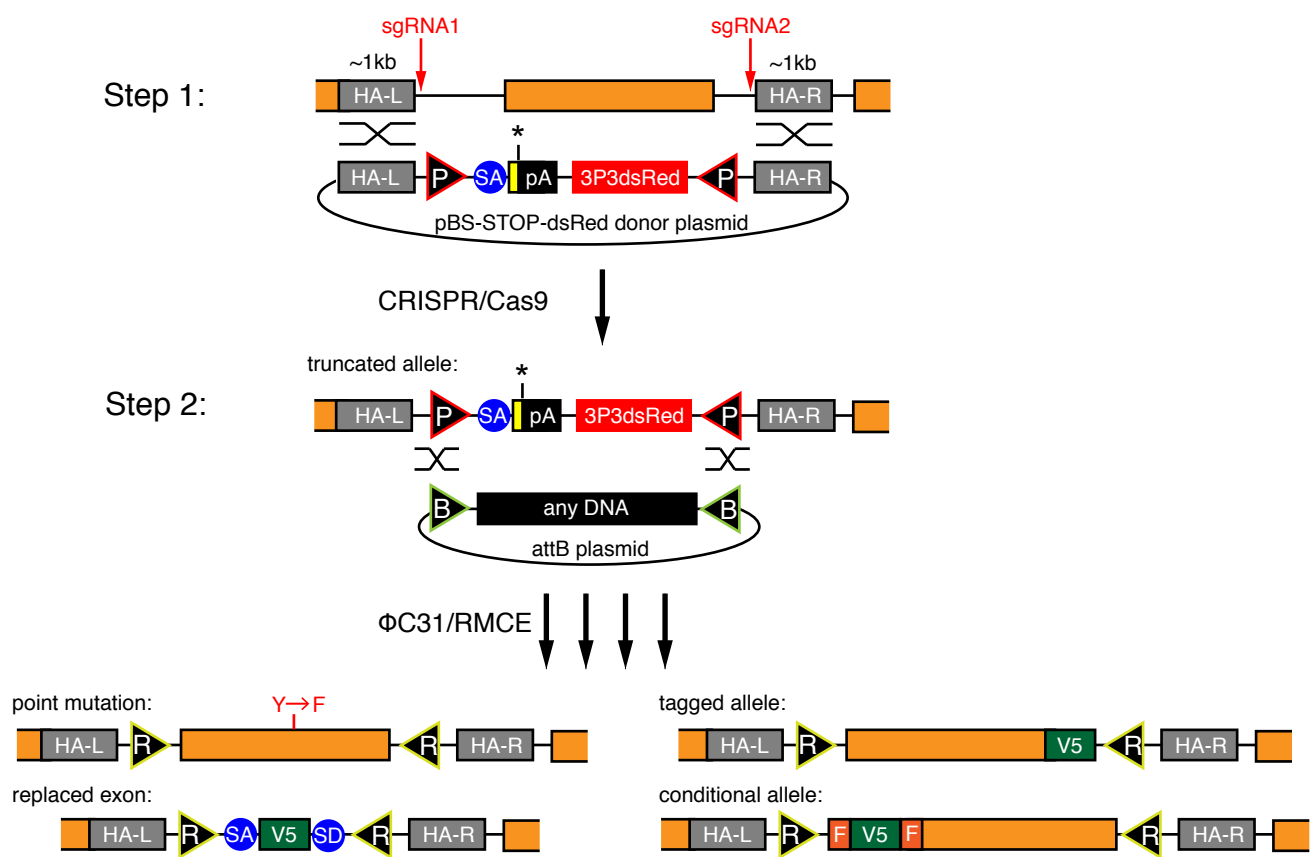
(a) Lethality assay of the *salm*-dsRed alleles as homozygous or in trans to *salm*[1]. (b) All tagged *salm*[1st intron] alleles regain homozygous viability after step 2. (c-g) Localisation of the tagged Salm proteins. Untagged Salm is located in the nucleus of wild type IFMs (c, e), whereas V5 tagged Salm is only detected in the *salm*[1st intron-TY1-V5] and the *salm*[1st intron-FRT-TY1-FRT-V5] alleles (d, g). FLAG is found in the IFM nuclei of *salm*[1st intron-GFP-FLAG] adults (f). The Salm-FRT-TY1-FRT-V5 protein is found in the nuclei (red arrow heads) and also located in dots in the cytoplasm (g). Note the normal fibrillar morphology of the myofibrils in all the homozygous *salm*[1st intron] alleles (d, f, g). Actin was stained with phalloidin and the scale bars are 10 µm.

Figure 6 | Engineering of the *bent* gene.

The entire genomic *bent* organisation is shown at the top with a 10 kb zoom-in below. Coding exons are in orange. The sgRNA targeting sites flanking exon 11 are indicated by red arrows and the resulting *bent*[11th exon-dsRed] allele is shown at the bottom.

Table 1 | Summary of the transformation efficiencies for the four different loci modified in this study.

Supplementary Table 1 | All primer sequences used in this study.



Zhang et al. Figure 1

CRISPR- and RMCE-assisted genome engineering timeline

1. CRISPR design and cloning ~ 10 days

- 1.1 Targeting strategy and sequencing of CRISPR target area in used fly strain - 2 days
- 1.2 sgRNA design and cloning of 1 kb donor homology arms into pJET - 2 days
- 1.3 sgRNA in vitro transcription and Golden Gate assembly of donor plasmid - 2 days
- 1.4 sgRNA activity assay in S2-cells - 2 days

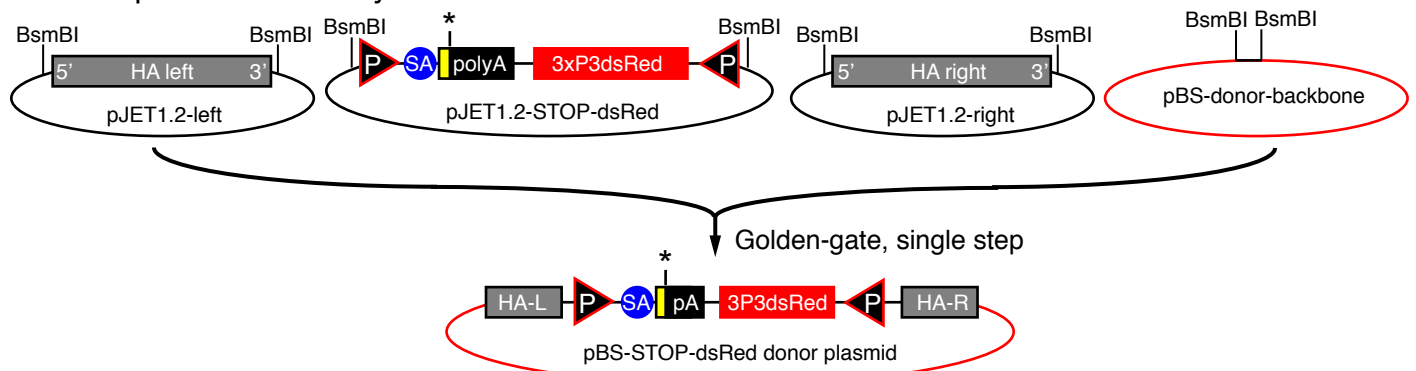
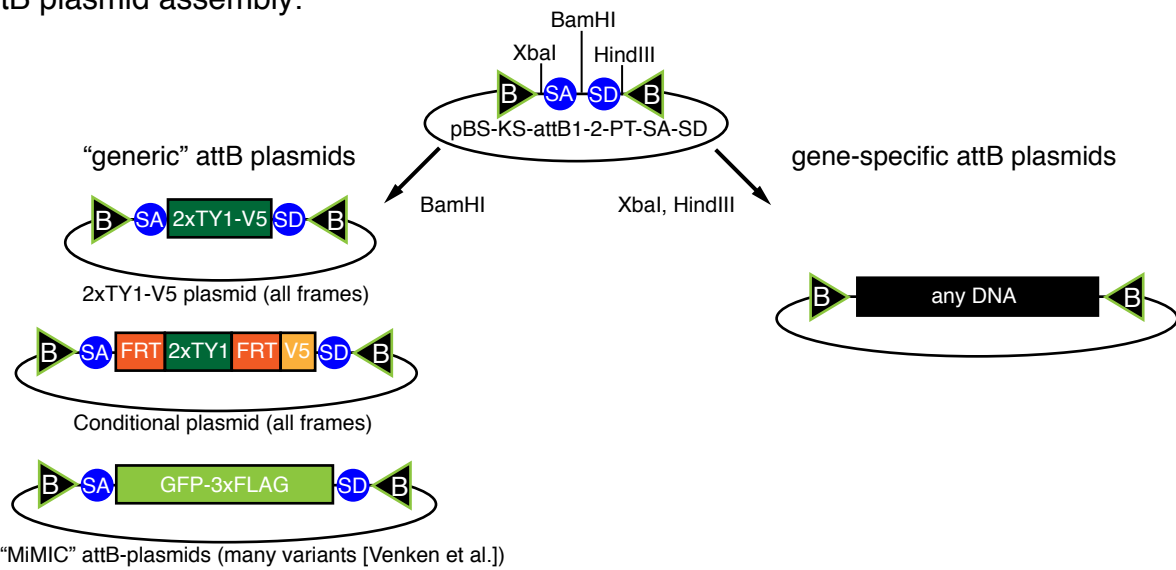
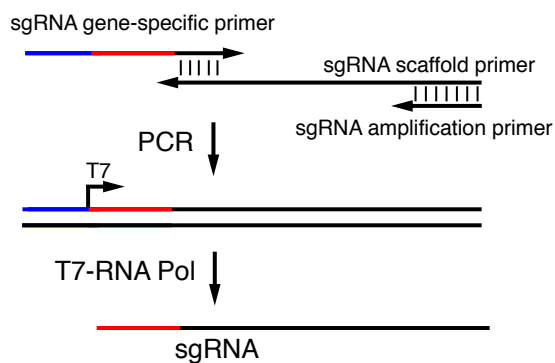
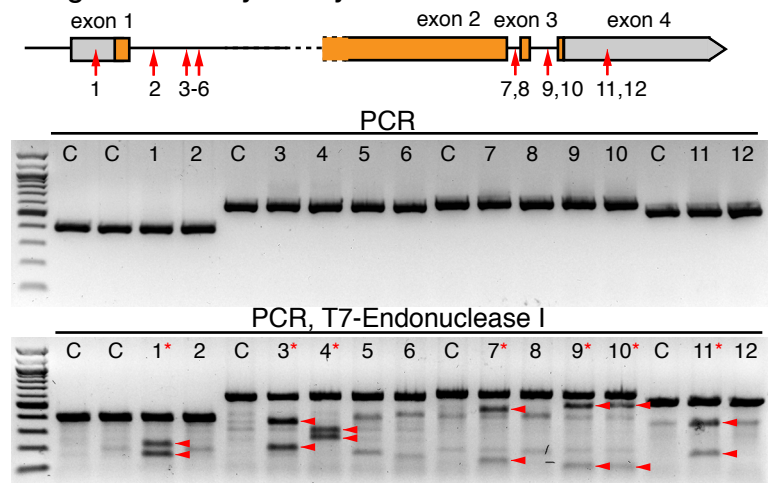
2. Fly step 1 - CRISPR-mediated HDR ~ 6 weeks

- 2.1 sgRNAs and donor vector injection into 600 - 800 *Act-Cas9, lig4[169]* embryos resulting in more than 50 fertile mosaic G_0 flies - 12 days
- 2.2 G_0 fly crosses to balancer and screen F_1 progeny for fluorescent red eyes - 12 days
- 2.3 Verification of positive F_1 s by PCR and sequencing, and generation of stocks - 12 days

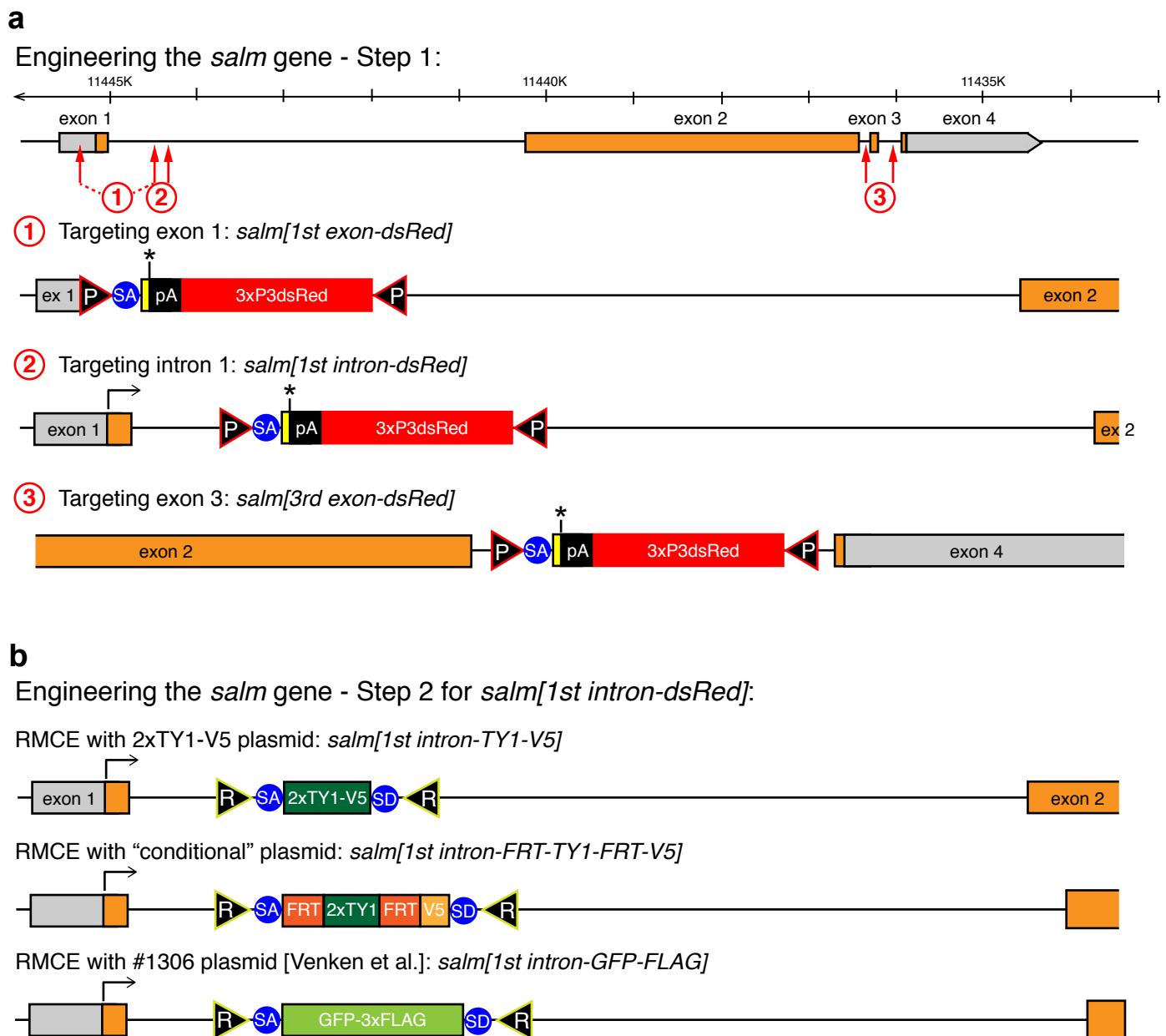
3. Fly step 2 - Φ C31-mediated RMCE ~ 6 weeks

- 3.1 Injection of "generic" or gene-specific attB plasmid together with nos Φ C31 plasmid into ~ 200 embryos from stocks generated at 2.3 - 12 days
- 3.2 G_0 fly crosses to balancer and screen F_1 progeny for non-fluorescent eyes - 12 days
- 3.3 Verification of positive F_1 s by PCR and generation of final stocks - 12 days

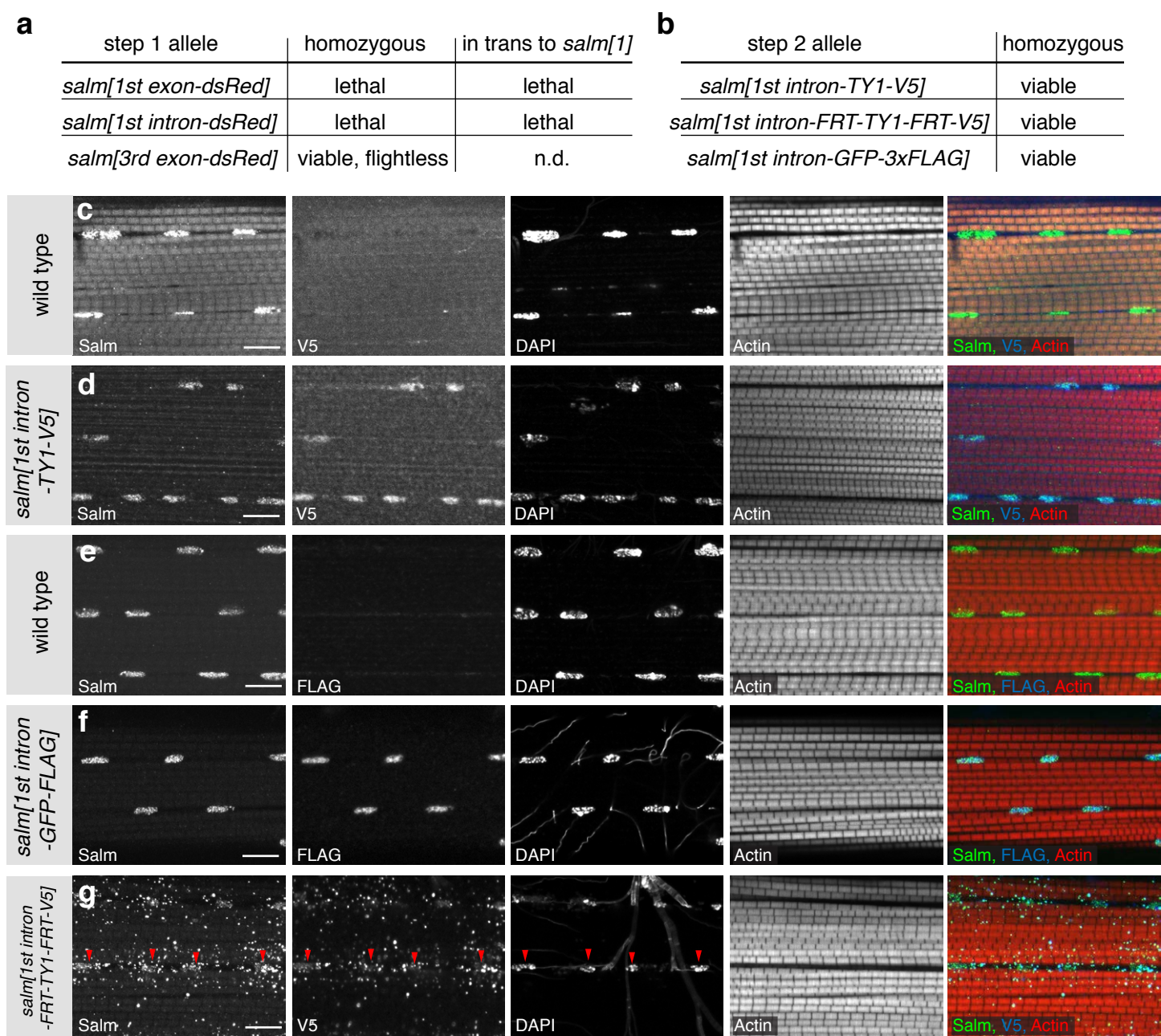
Zhang et al. Figure 2

a Donor plasmid assembly:**b** attB plasmid assembly:**c** sgRNA synthesis:**d** sgRNA activity assay:

Zhang et al. Figure 3

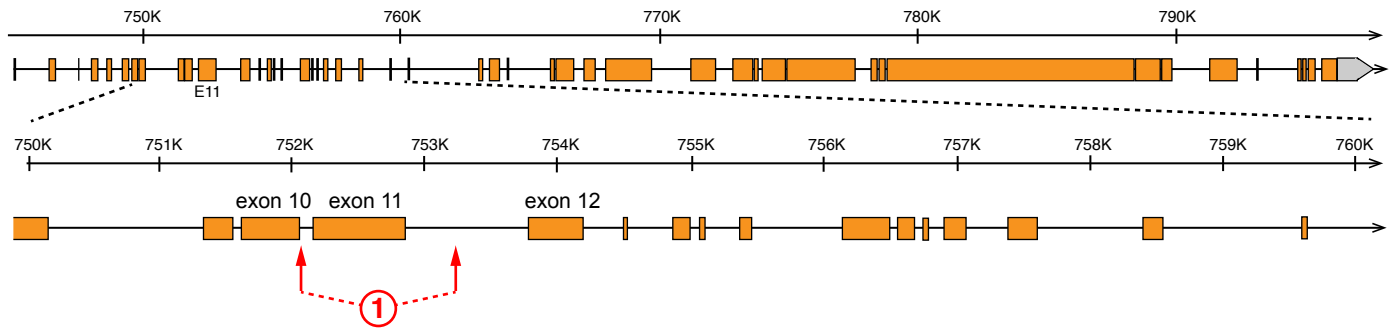


Zhang et al. Figure 4



Zhang et al. Figure 5

bent genomic:



CRISPR/Cas9

① Targeting exon 11: *bent*[11th exon-dsRed]



Zhang et al. Figure 6

Locus	Injected genotype	Injected embryos	Larvae	G0 adults	Fertile G0 adults	Independent F1 carriers	Total red eyed F1	Independent carriers per fertile G0s
<i>salm</i> 1st exon	<i>Lig4</i> -/-	1500	396	166	n.d.	0	0	n.d.
<i>salm</i> 1st intron	<i>Lig4</i> -/-	700	171	149	n.d.	2	11	n.d.
<i>salm</i> 3rd exon	<i>Lig4</i> -/-	1500	312	150	n.d.	4	72	n.d.
<i>salm</i> 1st exon	<i>Act5C-Cas9, Lig4</i> -/-	700	124	48	30	1	13	3,3%
<i>salm</i> 1st intron	<i>Act5C-Cas9, Lig4</i> -/-	700	200	122	64	9	54	14,1%
<i>salm</i> 3rd exon	<i>Act5C-Cas9, Lig4</i> -/-	700	291	150	99	4	59	4,0%
<i>bent</i> 11th exon	<i>Act5C-Cas9, Lig4</i> -/-	700	204	118	56	1	5	1,8%

Zhang et al. Table 1

Application	Primer ID	Primer name	sequence
STOP-dsRed donor	XZ82	attp1-SA forward-pCL1	CACACCAGGTCTCA ctog AAGCTTCCCAGGTGAGAAG
	XZ83	attp1-SA reverse-pCL1	CACACCAGGTCTCA ggtgTTAGTTAGTTAGACTGCGG
	XZ84	sv40 forward-pCL1	CACACCAGGTCTCA caccGCGATCCAGACATGAT
	XZ85	sv40 reverse-pCL1	CACACCAGGTCTCA gccagACTAGTTGATCATA ATCAGCCA
	XZ86	3p3-DsRed-SV40 forward-	CACACCAGGTCTCA tggcTCGCCCGGGGATCTAA
	XZ87	3p3-DsRed-SV40 reverse-pCL1	CACACCAGGTCTCAatttTCACACCCGATATGCC
	XZ88	attP2 forward-pCL1	CACACCAGGTCTCAaata TTCAACCCCTTGTGTCATGTCGG
	XZ89	attP2 reverse-pCL1	CACACCAGGTCTCAttccGCGGCCAGGTGAGAAG
	XZ195	STOP-dsRed forward	ACGTCTCA ccagTCTCGAAGCTTCCAGGTC
	XZ196	STOP-dsRed reverse	ACGTCTCA aacaGTCTCTCGTTCGCGGCG
<i>salm</i> exon 1	XZ142	exon 1 left arm fragment 1 sense	CACACCACGTCTCA ggacGGGAGCACCATACACA
	XZ143	exon 1 left arm fragment 1 antisense	CACACCACGTCTCA atagACGTTTATGAACTTGTTCGG
	XZ144	exon 1 left arm fragment 2 sense	CACACCACGTCTCA ctatCAGGTAAACAGCGACTGC
	XZ145	exon 1 left arm fragment 2 antisense	CACACCACGTCTCA ctggCCTTTGCGCGTGTTTA
	XZ148	exon 1 right arm sense	CACACCACGTCTCA tgttAGGCAATTTAATACAAATTCAAA
	XZ149	exon 1 right arm antisense	CACACCACGTCTCA agaaTTGGCTTTGCACTGAC
<i>salm</i> intron 1	XZ152	intron 1 left arm sense	CACACCACGTCTCA ggacGACTTCTGCGGCTGT
	XZ153	intron 1 left arm antisense	CACACCACGTCTCA ctggCCTCTGATGCCACAAT
	XZ154	intron 1 right arm sense	CACACCACGTCTCA tgttGGACATATCTATACATTTAAACCAT
	XZ155	intron 1 right arm antisense	CACACCACGTCTCA gcatTAAGTTATGCGAGCGC
<i>salm</i> exon 3		Oligo Name	Sequence
	XZ157	exon 3 left arm sense	CACACCACGTCTCA CGGACTGCGGCGAGGACTACG
	XZ158	exon 3 left arm antisense	CACACCACGTCTCA CTGGAAACCCCAATAAATTCAG
	XZ159	exon 3 right arm sense	CACACCACGTCTCA TGTGGCCCTAATGACCATCTT
	XZ160	exon 3 right arm antisense	CACACCACGTCTCA GCACATTCCAATAGATTATTAACGTG
<i>bent</i> exon 11	wk049	PCR left homology arm 1	aacgtctctGTATctagccgtcaaataggtctctgg
	wk050	PCR left homology arm 1	agcgctctctgcttgggtggcatatagcacTC
	wk051	PCR right homology arm 1	tgcgtctcaaacggcacgttctctgcacttcttgg
	wk052	PCR right homology arm 1	gtcgtctctACACggtattggcggaagagcagc
pBS-backbone	XZ150	pBS-GGAC-TTCT sense	CACACCACGTCTCA ttctGCAGGTGGAGCTCCAGCTTT
	XZ151	pBS-GGAC-TTCT antisense	CACACCACGTCTCA gtccGTACCCCAATTCGCCCT
	XZ156	pBS-GGAC-ATGC sense	CACACCACGTCTCA atgcAGGTGGAGCTCCAGCTTT
	XZ151	pBS-GGAC-ATGC antisense	CACACCACGTCTCA gtccGTACCCCAATTCGCCCT
	XZ161	pBS-CGGA-GTGC sense	CACACCACGTCTCA gtgcAGGTGGAGCTCCAGCTTT
	XZ162	pBS-CGGA-GTGC antisense	CACACCACGTCTCA tccgTACCCAATTCGCCCT
<i>salm</i> sgRNAs	sgRNA1	sgRNA1 gene-specific targeting oligo	TAATACGACTCACTATAG TGGGAAACGCGTAGTACCGC GTTTTAGAGCTAGAAATAGC
	sgRNA2	sgRNA2 gene-specific targeting oligo	TAATACGACTCACTATAG GCACTTTTGTGTTTTGCCGT GTTTTAGAGCTAGAAATAGC
	sgRNA3	sgRNA3 gene-specific targeting oligo	TAATACGACTCACTATAG AGAGGCGAGAAATCGTAG GTTTTAGAGCTAGAAATAGC
	sgRNA4	sgRNA4 gene-specific targeting oligo	TAATACGACTCACTATAG CAGTTTTCCCGCCGATTATA GTTTTAGAGCTAGAAATAGC
	sgRNA5	sgRNA5 gene-specific targeting oligo	TAATACGACTCACTATAG CAAACGTTAACAGCTTCTAT GTTTTAGAGCTAGAAATAGC
	sgRNA6	sgRNA6 gene-specific targeting oligo	TAATACGACTCACTATAG CAAGATCGCAAAAGGGGCGC GTTTTAGAGCTAGAAATAGC
	sgRNA7	sgRNA7 gene-specific targeting oligo	TAATACGACTCACTATAG TTTATTGGGGGGTTTTCTAA GTTTTAGAGCTAGAAATAGC
	sgRNA8	sgRNA8 gene-specific targeting oligo	TAATACGACTCACTATAG ATTTAAACGAGAAACTGAT GTTTTAGAGCTAGAAATAGC
	sgRNA9	sgRNA9 gene-specific targeting oligo	TAATACGACTCACTATAG AAGATGGTCATTAGGGGCAT GTTTTAGAGCTAGAAATAGC
	sgRNA10	sgRNA10 gene-specific targeting oligo	TAATACGACTCACTATAG TATTTAATAAGATGGTCATT GTTTTAGAGCTAGAAATAGC
	sgRNA11	sgRNA11 gene-specific targeting oligo	TAATACGACTCACTATAG CAAGTTTTAGAGCGAAATGA GTTTTAGAGCTAGAAATAGC
	sgRNA12	sgRNA12 gene-specific targeting oligo	TAATACGACTCACTATAG CACGTACACCCATACTAAGG GTTTTAGAGCTAGAAATAGC
<i>bent</i> sgRNAs	sg_1	sgRNA1 gene-specific targeting oligo	TAATACGACTCACTATAG ACCAGTCGTTCTCTGTTATA GTTTTAGAGCTAGAAATAGC used
	sg_2	sgRNA2 gene-specific targeting oligo	TAATACGACTCACTATAG AATTCGAACCCAAGTGTACC GTTTTAGAGCTAGAAATAGC negative in S2 cells
	sg_3	sgRNA3 gene-specific targeting oligo	TAATACGACTCACTATAG ACAATTATCGAATTAATCACT GTTTTAGAGCTAGAAATAGC used
	sg_4	sgRNA4 gene-specific targeting oligo	TAATACGACTCACTATAG GCAGGCTCTAATTTCTAATG GTTTTAGAGCTAGAAATAGC negative in S2 cells
	XZ100	scaffold oligo	AAAAGCACCAGCTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTATTTTAACTTGCTATTTCTAGCTCTAAAAAC
	XZ101	antisense primer for sgRNA production	AAAAGCACCAGCTCGGTGCC
sgRNAs into CO6-U6-gRNA_hsp70-Cas9			
	XZ123	sgRNA1 sense oligo	CTTCGTGGGAAACGCGTAGTACCGC
	XZ124	sgRNA1 antisense oligo	AAACGCGGTACTACGCTTTCCCA
	XZ125	sgRNA3 sense oligo	CTTCGAGAGGCGAGAAATCGTAGGT
	XZ126	sgRNA3 antisense oligo	AAACACCTACGATTTCTGCCTCTG
	XZ127	sgRNA4 sense oligo	CTTCGAGTTTTCCCGCCGATTATA
	XZ128	sgRNA4 antisense oligo	AAACTATAATCGGGGGAAACTG
	XZ129	sgRNA7 sense oligo	CTTCGTTTTATTGGGGGGTTTTCTAA
	XZ130	sgRNA7 antisense oligo	AAACTTAGAAAACCCCAATAAA
	XZ131	sgRNA9 sense oligo	CTTCGAAGATGGTCATTAGGGGCAT
	XZ132	sgRNA9 antisense oligo	AAACATGCCCTAATGACCATCTT